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Uncoupling protein expression in skeletal muscle and adipose tissue in response to *in vivo* porcine somatotropin treatment☆

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Abstract

These experiments examined the potential roles of somatropin (pST) and IGF-I in the regulation of uncoupling protein (UCP)2 and UCP3 and their regulatory proteins peroxisome proliferator activated receptor (PPAR) α, γ and δ using *in vivo* pST treatment of swine and *in vitro* supplementation of pST or IGF-I to adipose slices. Six, 90 kg barrows were treated with recombinant pST (10 mg) for 2 week while another six pigs were injected with buffer. Total RNA from outer subcutaneous adipose (OSQ) and middle subcutaneous adipose (MSQ) tissues, leaf fat, liver and longissimus (LM) was amplified by reverse transcription-PCR with quantification of transcripts by capillary electrophoresis with laser-induced fluorescence detection. UCP2 mRNA abundance increased in liver ($P<0.001$) and all three adipose tissues by pST treatment ($P<0.05$). Administration of pST increased UCP3 mRNA abundance by 42% in LM ($P<0.01$). PPARα mRNA abundance increased with pST treatment by 29% in liver ($P<0.05$), while decreasing 25% in LM ($P<0.05$). PPARγ mRNA abundance decreased 32% ($P<0.01$) while PPARδ increased 48% in LM ($P<0.01$) with pST administration. *In vitro*, pST reduced UCP2 mRNA abundance in OSQ and MSQ tissue slices ($P<0.05$). UCP3 mRNA abundance decreased in OSQ ($P<0.05$) but increased in MSQ ($P<0.05$) with pST. In contrast, IGF-I increased UCP2 and UCP3 mRNA abundance in both MSQ and OSQ slices ($P<0.05$). These experiments suggest pST, IGF-I and metabolic adaptations to pST contribute to regulating UCP2 and UCP3.
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Keywords: Uncoupling protein; PPAR; Somatotropin; IGF-I; Swine

1. Introduction

The uncoupling proteins (UCPs) have been grouped into a family of proteins based upon their *in vitro* capacity to uncouple mitochondrial respiration [1]. Uncoupling protein 1 was the first of these proteins identified and shown to contribute to non-shivering thermogenesis [2], but is not expressed in swine due to the absence of brown adipose tissue [3]. Other member of this family, UCP2 and UCP3, share 55% and 57% amino acid identity with UCP1 and 73% with each other [4,5]. However, UCP2 and UCP3 demonstrate much weaker uncoupling activity [6] and through a number of studies have been shown to have primary functions independent of a significant role in uncoupling activity and heat production [7,9,10]. Uncoupling protein 2 has been associated with metabolism of free radicals and mild uncoupling activity [5,7]. Using UCP3 knockout mice, UCP3 has been associated with changes in mitochondrial energy production that suggests some minor uncoupling activity specific to skeletal muscle [8] while the majority of studies have

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indicated a more specific role for UCP3 with regulation of fatty acid metabolism and ATP-dependent processes [9,10]. Damon et al. [11] and Spurlock et al. [12] have reported that both UCP2 and UCP3 mRNA are detected in porcine skeletal muscle and adipose tissue.

Hormonal regulation of these genes has been demonstrated *in vitro*, with somatotropin (GH) reducing uncoupling protein UCP2 and UCP3 mRNA abundance in adipose tissues slices from finishing pigs [13]. However the potential role of GH in the regulation of the uncoupling proteins in other major metabolic tissues of the pig has not been examined. Somatotropin is a potent inducer of lipid metabolism [14] as well as affecting free radical metabolism [15]. Since the uncoupling proteins are believed to have roles in these processes, GH may alter UCP expression in peripheral tissues *in vivo* through an indirect mechanism.

For the *in vivo* situation, the interaction of hormones in the endocrine milieu is important for overall metabolic regulation. Perhaps due to the variety of endocrine interactions *in vivo*, the potential role of GH in the regulation of the uncoupling proteins within adipose tissue is unclear [16–18]. Any and all *in vivo* studies with GH can be confounded by the known impact of GH on insulin-like growth factor I (IGF-I) secretion and the subsequent actions of IGF-I on peripheral tissues. The present study was designed to determine if the porcine uncoupling proteins are regulated *in vivo* by somatotropin, a modifier of energy metabolism and secondly to determine if IGF-I has a role in regulation of the uncoupling proteins in swine. The overall metabolic effects of GH on peripheral metabolism would suggest that UCP expression would be increased in peripheral tissues with GH treatment through its actions on IGF-I secretion and fatty acid metabolism. Furthermore, peroxisome proliferator activated receptor (PPAR) α, δ and γ mRNA abundance were analyzed because of their suggested role in the regulation of UCP gene expression [19–21] in an attempt to identify a mechanism for any changes in UCP mRNA abundance.

2. Materials and methods

Twelve crossbred barrows (Yorkshire × Landrace), weighing 70 kg, were individually penned in environmentally controlled housing. Animals were individually fed a basal diet containing 18% CP, 1.2% lysine, and 3.5 Mcal of DE/kg *ad libitum* as previously reported [22].

At 90 kg, six randomly selected pigs were treated with daily injections of sterile recombinant pST (10 mg; Southern Cross Biotech, Toorak, Victoria, Australia) in sodium bicarbonate buffer (pH 9.4). This dosage was based upon previous studies in pigs of this size [23,24]. The other six pigs served as controls and were injected with sterile bicarbonate buffer alone. Injections (1.0 mL) were performed into the extensor neck muscles between 08:00 and 08:30. Feed was presented at 09:00. With initiation of pST treatment, the feed was offered at 85% of calculated *ad libitum* intake [25], based upon BW and adjusted every 3 d. Animals were maintained on treatment for 2 week. A blood sample was obtained from each pig on d 14 of treatment at 15:00, 6 h after feed presentation. Animals were euthanized on d 15 at 08:00.

Various tissues were acquired following euthanasia by electrical stunning and exsanguination according to procedures approved by the Institutional Animal Use and Care Committee. Dorsal subcutaneous adipose tissue samples were collected from between the second and fourth thoracic vertebrae and subsequently outer subcutaneous adipose (OSQ) and middle subcutaneous adipose (MSQ) layers were separated at the connective tissue fascia separating the layers according to Anderson et al. [26], diced and frozen in liquid nitrogen. In addition, samples of liver, leaf (perirenal) fat and longissimus (LM) muscle were collected, diced and frozen in liquid nitrogen. Outer and middle subcutaneous adipose tissues were separated for analysis because of their known differences in metabolic activity, while leaf fat has a different metabolic profile from either subcutaneous adipose tissue [26]. The LM was selected as it represents a large, economically important muscle that responds to pST [27].

2.1. Hormone and metabolite analysis

Blood samples were centrifuged at 600 × *g* and serum samples were collected and stored at −70 °C for later analyses of hormones and metabolites. Concentrations of T_3 and cortisol were determined by homologous RIA using commercial kits (Diagnostics Products Co., Los Angeles, CA). Intraassay CV was 4.9% for T_3 and 3.4% for cortisol. Interassay CV was 6.6% for T_3 and 5.2% for cortisol. Serum insulin was measured using a homologus RIA kit with human standards (Linco Research Inc., St. Charles, MO). Dilutions of both the quality control solution and pig serum pool dilutions were parallel to the standard curve. Recovery of known amounts of unlabeled insulin yielded an average recovery of 97.6% of the added amount. Intraassay CV for insulin was 7.0%, while the interassay CV was 9.4%. Serum IGF-I was acid–ethanol extracted to remove binding proteins and then assayed using a heterologous immunoradiometric kit (Diagnostics Systems Laboratory Inc., Webster, TX) which was previously validated for swine [28]. Intrassay CV for IGF-I was 6.1% while the interassay CV was

3.7%. Serum glucose (Thermo DMA, Louisville, CO), triglycerides (DMA, Arlington, TX) and non-esterified free fatty acids (WakoChemical Co., Richmond, VA) were determined with kit-based assays.

2.2. Gene expression analyses by reverse transcription-PCR

Total RNA was isolated using TRI Reagent according to the manufacturer's protocol (Sigma–Aldrich, St. Louis, MO). Integrity of RNA was assessed via agarose gel electrophoresis and RNA concentration was determined spectrophotometrically using A260 and A280 measurements. Reverse transcription (RT) reactions (20 μL) consisted of 1 μg total RNA, 50 U SuperScript II reverse transcriptase (Invitrogen/Life Technologies, Carlsbad, CA), 40 U of an RNAse inhibitor (Invitrogen/Life Technologies), 0.5 mmol/L dNTP, and 100 ng random hexamer primers. Polymerase chain reaction was performed in 25 μL containing 20 mmol/L Tris–HCl, pH 8.4, 50 mmol/L KCl, 1.0 μL of the RT reaction, 1.0 U of Platinum Taq DNA polymerase (Hot Start, Invitrogen/Life Technologies), 0.2 mmol/L dNTP, 2.0 mmol/L Mg^{2+} (Invitrogen/Life Technologies), 10 pmol each of the UCP2, UCP3, PPARα, PPARδ, PPARγ and acyl CoA oxidase (ACOX) specific primers and 10 pmol of an appropriate mixture of primers and competimers specific for 18S rRNA (QuantumRNA Trade Mark Universal 18S Internal Standard; Ambion, Inc.; Austin, TX). Thermal cycling protocol for UCP2 and UCP3 was as follows: 1 cycle 94 °C for 2 min, followed by 35 cycles, 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min with a final extension at 72 °C for 8 min. Cycling protocol for PPARα, PPARγ and ACOX was as follows: 1 cycle 94 °C for 2 min, followed by 35 cycles, 94 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min with a final extension at 72 °C for 8 min. The thermal cycling protocol for PPARδ was derived from Lord et al. [29]. PCR amplifications were performed in a 25 μL total volume which contained a 1 μL aliquot of the reverse transcriptase product, 15 pmol of forward and reverse primers, 0.2 mmol/L dNTP, 1.0 mmol/L Mg^2, 20 mmol/L Tris–HCl, pH 8.4, 50 mmol/L KCl, and 1.0 U of Platinum Taq DNA polymerase (Hot Start, Invitrogen/Life Technologies). The thermal cycling profile was as follows: 1 cycle 94 °C for 2 min, followed by 35 cycles, 94 °C for 1 min, 69 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 5 min.

The following primers were used for generating 210 base-pair PCR products corresponding to a portion of the pig UCP2 coding sequence: 5′-CTGCAGATCCAGGGAGAAAG-3′ (forward), 5′-GCTTGACGGAGTCGTAGAGG-3′ (reverse). The primers for UCP3 were used to generate a 200 base-pair product: 5′-ACGATGGATGCCTACAGGAC-3′ (forward), 5′-TCCGAAGGCAGAGACAAAGT-3′ (reverse). The PPARα primers were used to generate a 196 base-pair product: 5′-GGGATCAGATGGATCCGTTA-3′ (forward), 5′-AAAGAAGCCCTTGCAACCTT-3′ (reverse). The PPARδ primers generated a 358 base-pair product: 5-CCGCATGAAGCTGGAGTACGAG-3′ (forward), 5′-CTGCCACAACGTCTCGATGTCG-3′ (reverse) following the procedures of Lord et al. [29]. The PPARγ primers produced a 210 base-pair product: 5′-GCCCTTCACCACTGTTGATT-3′ (forward), 5′-GAGTTGGAAGGCTCTTCGTG-3′ (reverse), which encompasses both PPARγ1 and PPARγ2. The ACOX primers were used to generate a 214 base-pair product: 5′-CTCGCAGACCCAGATGAAAT-3′ (forward), 5′-AGCCTCGAAGATGAGTTCCA-3′ (reverse). Primers for PCR reactions were designed to span introns or intron–exon boundaries to exclude genomic DNA amplification and the specificity of the PCR reactions was further confirmed by agarose gel electrophoresis of the amplicons. The UCP2, UCP3, PPARα, PPARδ, PPARγ and ACOX amplicons were excised from an agarose gel, re-amplified, and run through a GenElute PCR clean-up kit (Sigma–Aldrich). The amplicons were subsequently sequenced to confirm identity using automated fluorescent DNA sequencing (ABI 310, Perkin-Elmer Applied Biosystems, Foster City, CA).

2.3. Capillary electrophoresis with laser-induced fluorescence detection

Aliquots (2 μL) of RT-PCR samples were diluted 1:100 with deionized water before capillary electrophoresis with laser-induced fluorescence detection (CE/LIF). A detailed description and validation of the CE/LIF technique used in this study for quantitative analysis of gene expression was reported previously [30]. Briefly, a P/ACE MDQ CE instrument (Beckman Coulter, Fullerton, CA) equipped with an argon ion LIF detector was used. Capillaries were 75 μm i.d. × 32 cm μSIL-DNA (Agilent Technologies, Folsom, CA). EnhanCE dye (Beckman Coulter) was added to the DNA separation buffer (Sigma–Aldrich) to a final concentration of 0.5 μg/mL. Samples were loaded by electrokinetic injection at 3.5 kV for 3.5 s and run in reverse polarity at 8.1 kV for 5 min. Integrated peak area for the PCR products separated by CE was calculated using P/ACE MDQ software (Beckman Coulter).

2.4. Quantification of mRNA abundance

The relative level of mRNA abundance was determined as the ratio of integrated peak area for each individual gene PCR product relative to that of a coamplified 18S internal standard (QuantumRNA Trade Mark Universal 18S Internal Standard; Ambion, Inc.). Values are presented as the mean ± S.E.M. of six individual determinations.

2.5. In vitro expression

For *in vitro* experiments, subcutaneous adipose tissue was collected from another four crossbred barrows (Yorkshire × Landrace) at approximately 110 kg bwt that were fed the previously described dietary protocol. Dorsal subcutaneous adipose tissue samples from between the second and fourth thoracic vertebrae were acquired following euthanasia by electrical stunning and exsanguination, according to procedures approved by the Institutional Animal Use and Care Committee. Adipose tissue was prepared for chronic tissue slice incubation according to methods previously described [13]. Briefly, dissected middle adipose tissue and outer subcutaneous adipose tissue were diced into 1 cm × 4 cm strips and placed in Hanks buffer (37 °C, pH 7.4). Adipose tissue strips were then dissected clean of any extraneous muscle tissue and further separated into 1 cm cubes in a laminar flow hood. Adipose tissue explants (approximately 100 mg) were prepared by slicing tissue cubes with a Stadie-Riggs microtome. Tissue slices (400 μm thickness) were rinsed twice with fresh Hanks buffer (37 °C, pH 7.4), blotted free of excess liquid and weighed. Tissue slices were then transferred to 12 well tissue culture plates containing 1 mL of DMEM/F12 with 25 mM Hepes, 0.5% BSA, pH 7.4 and the various hormone supplements of interest. Triplicate tissue slices were incubated with either basal medium or hormone supplemented media in a tissue culture incubator at 37 °C with 95% air/5% CO_2 for 24 h.

The same preparation of recombinant porcine somatotropin (pST) was used for the *in vitro* experiments as for the *in vivo* experiment. The pST was prepared fresh daily for the experimental trials in sodium bicarbonate buffer (pH 9.4). Serial dilutions of 10, 100 and 1000 ng/mL were supplemented to medium for 24 h incubations and compared to a basal medium (control). Additional cultures were incubated with recombinant human IGF-I which was prepared in 0.01N HCl (Sigma–Aldrich, St. Louis, MO). Serial dilutions of 10, 50 and 250 ng/mL were supplemented to medium for 24 h incubations for comparison to a basal medium (control).

2.6. Real-time PCR analysis of gene expression

The expression of UCP2 and UCP3 were assessed in tissue slices following 24 h of incubation with various concentrations of pST or IGF-I by real-time PCR analysis. Tissue samples from these incubations were blotted and transferred to microfuge tubes with subsequent freezing in liquid nitrogen and storage at −80 °C prior to analysis for uncoupling protein gene expression. Total RNA was isolated by methods mentioned above.

Reverse transcription (RT) and real-time PCR analysis were performed in a single tube using the QuantiTect SYBR Green RT-PCR protocol (Qiagen, Inc., Valencia, CA) according to procedures previously published by this laboratory [13]. Reactions (25 μL) consisted of 1 μg total RNA, 12.5 μL QuantiTect SYBR Green RT-PCR Master Mix, 0.5 μM primers, 0.25 μL QuantiTect RT Mix, 9 μL RNase-free H_2O. Thermal cycling and data acquisition were performed with a DNA Engine Opticon Continuous Fluorescence Detection System (MJ Research, Waltham, MA). Thermal cycling parameters were as follows: 1 cycle 50 °C for 30 min (reverse transcription), 1 cycle 95 °C for 15 min (PCR activation), followed by 30 cycles, 94 °C for 15 s, 58 °C for 30 s, 72 °C for 30 s, with a final extension at 72 °C for 8 min. Melting curve analysis was performed on all real-time PCR reactions to confirm specificity and identity of the real-time PCR products. A nontemplate control was run for every assay. Specificity of real-time PCR products was further confirmed by agarose gel electrophoresis. The one-step real-time PCR for UCP2, UCP3 and 18S were optimized for linearity (exponential amplification) from >20 to <30 cycles under the conditions described above and as previously reported [13].

The same primers were used for generating UCP2 and UCP3 amplicons as for the *in vivo* experiment. The primers for 18S ribosomal RNA were purchased (QuantumRNA Trade Mark Universal 18S Internal Standard; Ambion, Inc.; Austin, TX).

2.7. Quantification of gene expression

At the end of the real time-PCR, baseline and threshold crossing values (C_T) for UCP2, UCP3 and 18S were calculated using the Opticon Monitor Software (Version 1.06; MJ Research, Waltham, MA) and the C_T values were exported to Microsoft Excel for analysis. The relative expression of UCP2 and UCP3 mRNA, standardized against the amount of 18S rRNA, in adipose tissue explants was calculated using the $\Delta\Delta C_T$ method [31,32]. Values are presented as the mean ± S.E.M. of

Table 1
Serum hormone concentrations for barrows injected with buffer (control) or pST (10 mg/d) during a 2 week trial

	Control	pST	Significance (P)
IGF-I (ng/mL)	221 ± 21	824 ± 43	0.002
Insulin (μU/mL)	17.1 ± 2.6	92.6 ± 17.4	0.004
Cortisol (μg/dL)	3.3 ± 1.1	4.0 ± 1.4	0.699
Triiodothyronine (ng/L)	53.7 ± 7.2	90.3 ± 7.4	0.005

$n = 6$.

duplicate determinations from tissues from four individual animals.

2.8. Statistical analysis

Data were analyzed by one-way analysis of variance using SigmaStat software (SPSS Science, Chicago, IL) to test for treatment effects. Mean separation was analyzed using Student–Newman–Keuls test. Correlations between serum parameters and gene expression were evaluated with the Pearson product moment correlation calculated using the SigmaStat software. Means were defined as significantly different at $P < 0.05$.

3. Results

A previous report demonstrated that pST treatment elevated serum ST, produced a modest increase in serum leptin concentrations and also increased weight gain in these swine during the 2 weeks of the experiment [22]. Further analysis of serum from those swine demonstrated that serum IGF-I was elevated by three-fold with pST treatment (Table 1; $P < 0.01$) while insulin was increased four-fold ($P < 0.01$). In addition, serum T_3 was increased by 68% ($P < 0.01$). However, serum cortisol was unaffected by pST treatment ($P > 0.05$).

Serum glucose concentrations were not altered by pST treatment (Table 2; $P > 0.05$). Serum triglycerides were increased by 59% ($P < 0.05$) while serum NEFA were increased by 273% ($P < 0.01$) with pST treatment.

The UCP2 mRNA abundance was elevated by 81% in liver of pST treated swine relative to animals injected with buffer (Fig. 1a, $P < 0.001$). Abundance of UCP2 mRNA in all three sites of adipose tissue deposition was also elevated with pST treatment ($P < 0.05$). The muscle tissue examined in this study did not demonstrate a change in UCP2 mRNA abundance with pST treatment ($P > 0.05$ for LM).

Table 2
Serum metabolite concentrations for barrows injected with buffer (control) or pST (10 mg/d) during a 2 week trial

	Control	pST	Significance (P)
Glucose (mg/dL)	122 ± 10	138 ± 10	0.268
Triglycerides (mg/dL)	1.41 ± 0.16	2.24 ± 0.30	0.037
NEFA (μEq/L)	56.2 ± 4.6	209 ± 36.5	0.002

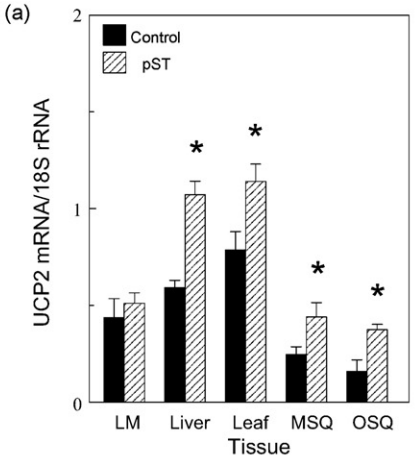
$n = 6$.

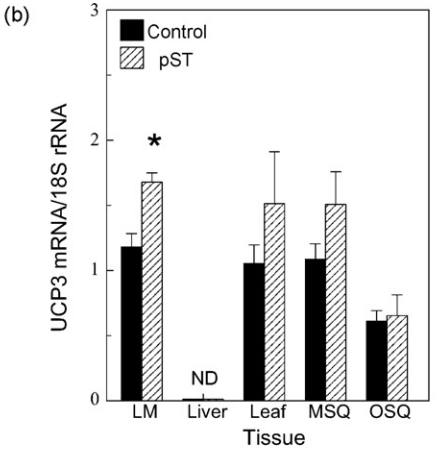
Uncoupling protein 3 mRNA abundance was 42% greater in LM of pST treated swine than control swine (Fig. 1b; $P < 0.01$). Abundance of UCP3 mRNA was similarly increased in leaf fat and MSQ, but variability between animals eliminated any difference between treatment groups ($P > 0.05$). Recombinant pST treatment did not alter UCP3 expression in OSQ ($P > 0.05$). Liver did not express detectable UCP3 mRNA.

Significant correlations ($P < 0.05$) were found between UCP2 mRNA abundance and peripheral hormones with metabolic functions (Fig. 1a). Serum somatotropin and leptin concentrations from these animals have been previously published [22] and were incorporated into the correlation analysis. Serum leptin was highly correlated with UCP2 mRNA abundance in leaf fat, but no other tissue. Somatotropin was correlated with UCP2 mRNA abundance in liver and OSQ. Some of the highest correlations were found between IGF-I and UCP2 mRNA abundance within liver and all of the adipose tissues, the tissues which demonstrated significant responses in UCP2 to pST treatment. Insulin concentration was highly correlated with hepatic UCP2 mRNA abundance. Triiodothyronine was positively correlated with UCP2 mRNA abundance in leaf fat only. No correlations were detected between the hormones examined and UCP2 mRNA abundance in LM.

Limited association between serum metabolites and UCP2 mRNA abundance was identified through correlation analysis (Fig. 1a). Serum glucose was correlated with UCP2 mRNA abundance in OSQ, but in no other tissue. Serum triglycerides were not associated with UCP2 mRNA abundance. However, serum NEFA were correlated with UCP2 mRNA abundance in liver and leaf fat.

Unlike UCP2, UCP3 mRNA abundance was correlated with the serum hormone and metabolite adaptations





Summary of significant correlations between serum hormone and metabolite levels and the abundance of UCP2 mRNA with pST treatment of barrows[1, 2, 3]

	Leptin[4]	Somatotropin[4]	IGF-I	Insulin	T3	Glucose	NEFA
			r (P)				
Liver	NC	0.615 (0.044)	0.864 (< 0.001)	0.939 (< 0.001)	NC	NC	0.747 (0.013)
Leaf Fat	0.828 (0.003)	NC	0.716 (0.020)	NC	0.782 (0.008)	NC	0.674 (0.046)
MSQ	NC	NC	0.605 (0.049)	NC	NC	NC	NC
OSQ	NC	0.674 (0.023)	0.722 (0.012)	NC	NC	0.602 (0.050)	NC

[1] Pearson's correlation coefficients (*r*) shown with P values in parentheses. NC = no correlation (P > 0.05).

[2] No correlations (P > 0.05) between LM, UCP2 and any serum parameter analyzed.

[3] No correlations (P > 0.05) between serum cortisol, T_4, glucose or triglycerides and UCP2.

[4] Serum hormone concentrations previously published (Journal of Animal Science 83:2501-2508, 2005).

Summary of significant correlations between serum hormone and metabolite levels and the abundance of UCP3 mRNA in longissimus with pST treatment of barrows[1, 2, 3]

	Somatotropin[4]	IGF-I	T_3	Triglycerides	NEFA
			r (P)		
LM	0.675 (0.016)	0.796 (0.002)	0.770 (0.003)	0.620 (0.032)	0.647 (0.031)

[1] Pearson's correlation coefficients (*r*) shown with P values in parentheses.

[2] No correlations (P > 0.05) between any assayed hormones/metabolites and UCP3 mRNA abundance in any other tissue examined.

[3] No correlations (P > 0.05) between serum leptin, insulin, cortisol, T_4, or glucose and UCP3 in LM.

[4] Serum hormone concentrations previously published (Journal of Animal Science 83:2501-2508, 2005)

Fig. 1. Relative UCP2 (a) and UCP3 (b) mRNA abundance in tissues from barrows following 14 d of pST administration (10 mg/d). Data are expressed as the mean ratio ± S.E.M. of specific UCP mRNA:18S rRNA for six pigs in each group. The asterisks indicate that the means differ from that of corresponding control (buffer injected) swine ($P < 0.05$, $n = 6$). Tables below the figures summarize the correlation analysis between UCP2 and UCP3 in the various tissues with serum hormones and metabolites. LM: longissimus; Leaf: leaf fat; MSQ: middle subcutaneous adipose tissue; OSQ: outer subcutaneous adipose tissue. ND: not detectable.

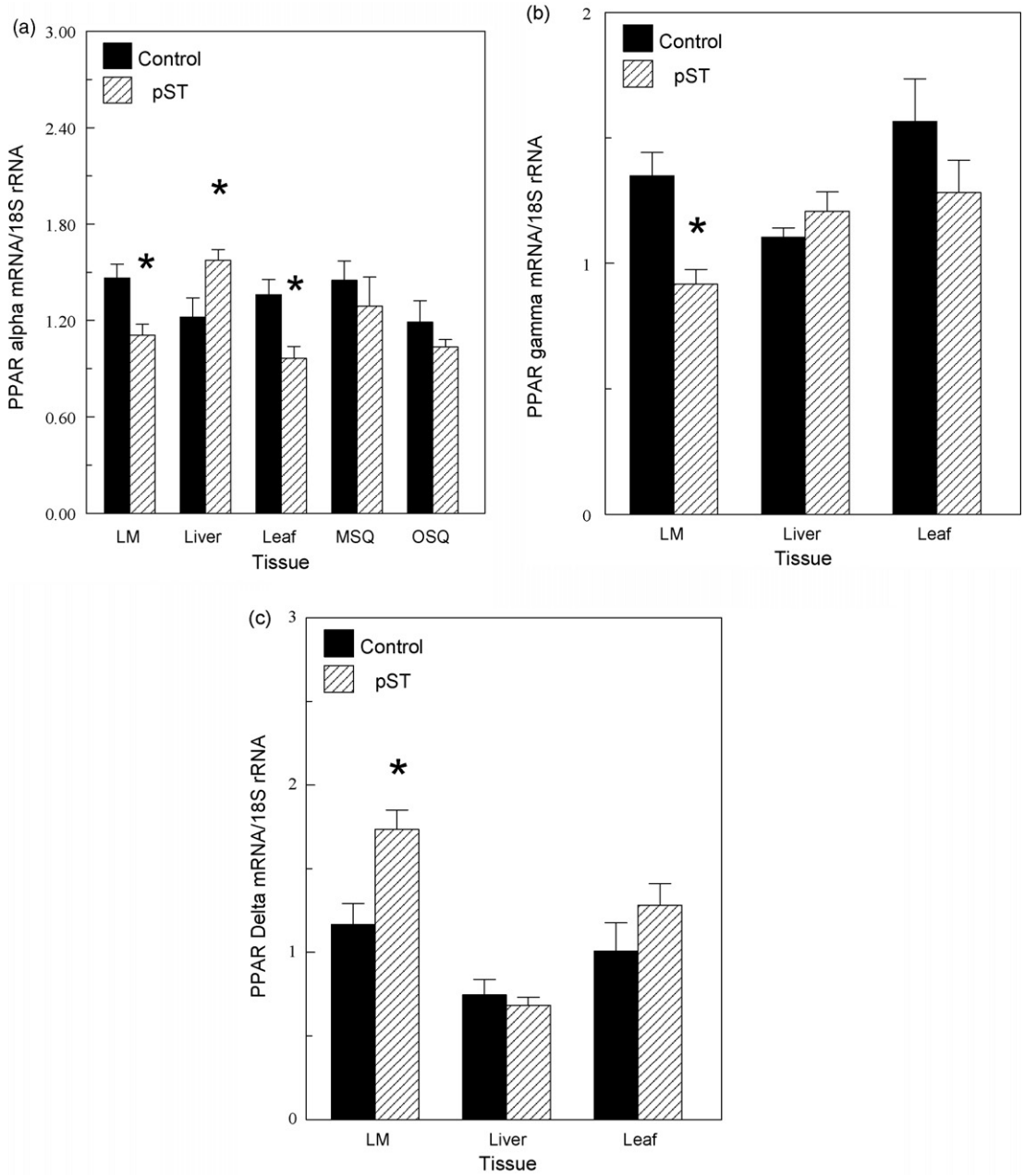


Fig. 2. Relative PPARα (a), PPARγ (b) and PPARδ mRNA (c) abundance in tissues from barrows following 14 d of pST administration (10 mg/d). Data are expressed as the mean ratio ± S.E.M. of specific PPAR mRNA:18S rRNA for six pigs in each group. The asterisks indicate that the means differ from that of corresponding control (buffer injected) swine ($P < 0.05$, $n = 6$). LM: longissimus; Leaf: leaf fat; MSQ: middle subcutaneous adipose tissue; OSQ: outer subcutaneous adipose tissue.

to somatotropin treatment only in LM, the only tissue to produce a response in UCP3 expression to pST treatment (Fig. 1b). Both IGF-I and T_3 were highly correlated with UCP3 mRNA abundance. Somatotropin was also positively correlated with UCP3 mRNA abundance. Uncoupling protein 3 mRNA abundance was correlated with serum triglycerides and NEFA in LM.

The relative abundance of PPARα was decreased 25% in longissimus ($P < 0.05$) and 30% in leaf fat ($P < 0.05$, Fig. 2a) by pST treatment. In contrast, PPARα mRNA abundance was increased by 29% in liver ($P < 0.05$). Treatment with pST had no effect on PPARα mRNA abundance in OSQ or MSQ ($P > 0.05$).

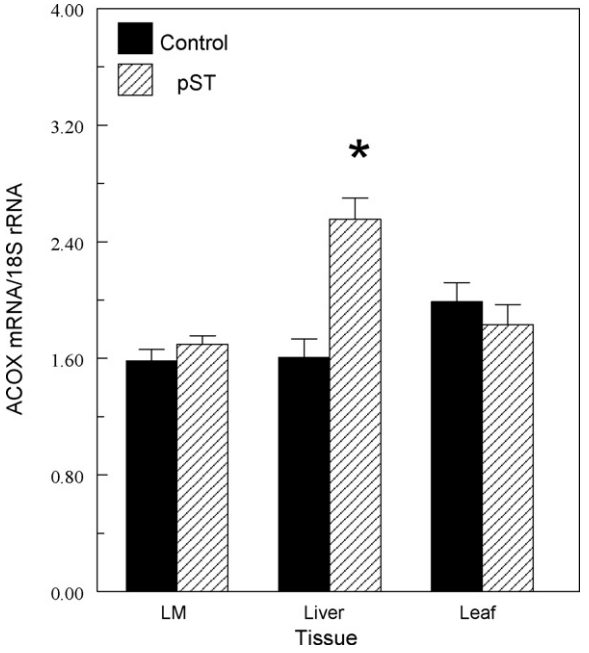


Fig. 3. Relative acyl CoA oxidase mRNA abundance in tissues from barrows following 14 d of pST administration (10 mg/d). Data are expressed as the mean ratio ± S.E.M. of ACOX mRNA:18S rRNA for six pigs in each group. The asterisks indicate that the means differ from that of corresponding control (buffer injected) swine ($P < 0.05$, $n = 6$). LM: longissimus; Leaf: leaf fat.

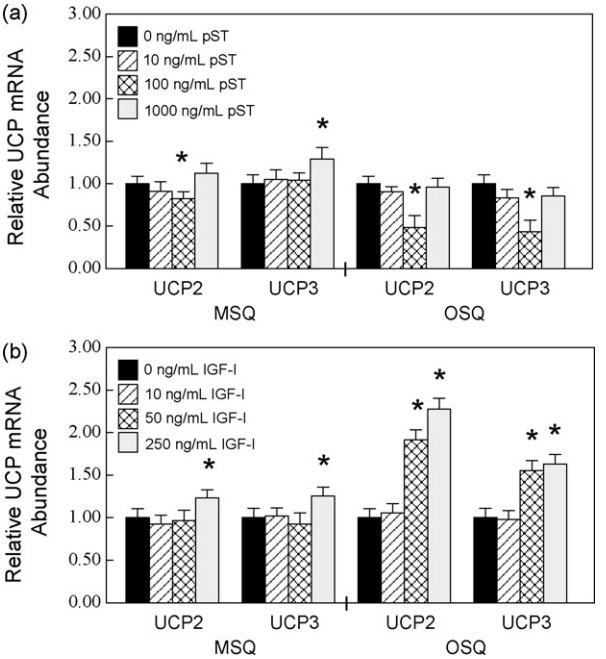


Fig. 4. Relative UCP2 and UCP3 mRNA abundance in subcutaneous adipose tissue explants in response to 24 h incubation with 0–1000 ng pST/mL medium (a) or with 0–250 ng human IGF-I/mL medium (b), followed by extraction for total RNA and subsequent real time-PCR analysis for UCP mRNA and 18S rRNA abundance. Data are expressed as the mean ± S.E. derived from the calculation of the $\Delta\Delta C_T$ for UCP and 18S of tissues from four pigs in each group. The asterisks indicate that the means differ from that of control (0 ng pST/mL) tissue incubations ($P < 0.05$, $n = 4$). MSQ: middle subcutaneous adipose; OSQ: outer subcutaneous adipose.

Those tissues which demonstrated a correlation between UCP mRNA abundance and serum free fatty acids were further analyzed for the abundance of PPARγ and PPARδ mRNA (Fig. 2b and c, respectively). The PPARγ mRNA abundance was reduced by 32% in LM with pST administration ($P < 0.01$). The PPARγ mRNA abundance in liver and leaf fat were unaffected by pST treatment ($P > 0.05$). The abundance of PPARδ mRNA was elevated in LM from pST treated swine relative to LM from saline injected controls ($P < 0.01$), while not affected in liver or leaf fat ($P > 0.05$).

Those tissues which demonstrated a change in PPARα were further examined for ACOX mRNA abundance. Liver ACOX mRNA abundance was increased with pST treatment of swine ($P < 0.05$, Fig. 3). However, neither LM or leaf fat ACOX mRNA abundance was affected by pST treatment ($P > 0.05$).

The incubation of adipose tissue slices with 100 ng pST/mL medium for 24 h resulted in a decrease in UCP2 in both MSQ and OSQ ($P < 0.05$, Fig. 4a). No other concentration of pST affected UCP2 mRNA abundance. The reduction in UCP2 mRNA abundance by pST in the OSQ was much greater than in the MSQ ($P < 0.05$). The abundance of UCP3 mRNA was increased in the MSQ with 1000 ng pST/mL medium ($P < 0.05$), with no effect of the other concentrations of pST. In contrast, UCP3 mRNA abundance was reduced by 100 ng pST/mL medium in the OSQ ($P < 0.05$).

Incubation with IGF-I produced an increase in UCP2 mRNA abundance at only the highest concentration (250 ng/mL) in the MSQ ($P < 0.05$, Fig. 4b), while both 50 and 250 ng IGF-I/mL medium produced large increases in UCP2 mRNA abundance in the OSQ relative to controls ($P < 0.05$). The pattern of UCP3 expression was similar. The UCP3 mRNA abundance was increased with 250 ng IGF-I/mL medium in the MSQ ($P < 0.05$), while both 50 and 250 ng IGF-I/mL medium produced increases in UCP3 mRNA abundance ($P < 0.05$).

4. Discussion

The present study has confirmed the presence of UCP2 and UCP3 mRNA in porcine skeletal muscle and adipose tissues, as previously demonstrated by Damon et al. [11] and Spurlock et al. [12]. This study has added new information demonstrating that pig UCPs

can be hormonally regulated *in vivo* by somatotropin. Somatotropin treatment of finishing swine altered UCP2 mRNA abundance in the various adipose tissues and liver, but did not affect expression in the skeletal muscle examined. In contrast, only UCP3 expression in skeletal muscle was affected by peripheral somatotropin treatment.

The question may be raised that the 15% dietary restriction altered the UCP expression in the controls, which might alter detection and interpretation of a pST effect. However, Spurlock et al. [12] demonstrated that UCP2 and UCP3 mRNA abundance in adipose tissue was not significantly altered by fasting for 96 h ($P > 0.05$). Therefore it is unlikely that a 15% dietary restriction will impact UCP expression in adipose tissue. Secondly, liver or skeletal muscle UCP2 and UCP3 mRNA abundance does not differ between these restricted fed animals and *ad libitum* fed animals from our other studies (unpublished observations).

The *in vivo* responses by UCP2 and UCP3 in the present study could be through the direct action of somatotropin on peripheral tissues or an indirect action through alterations in overall metabolic and hormonal profile. As reported previously and reproduced in the present study, somatotropin injection in swine produces an increase in serum IGF-I [33], insulin [34] and T_3 [35,36]. While the potential contributions of these various hormones to uncoupling protein regulation have been examined in other species [37,38], their relative role in swine is unknown.

Hioki et al. [18] reported no change in adipose UCP2 mRNA abundance in lean mice injected with somatotropin, but this may reflect a species variation. In contrast, somatotropin reduced UCP2 mRNA abundance in porcine subcutaneous adipose tissue *in vitro* [13]. That has been the only experiment to examine the potential actions of somatotropin on the expression of porcine UCP2. The present *in vivo* experiment does not support that previous *in vitro* study [13], suggesting that the *in vivo* stimulation of UCP2 with pST treatment may be indirect through the hormonal adaptations to elevation in serum somatotropin.

This conflict between the previous *in vitro* and the current *in vivo* data led to the *in vitro* dose response experiments with pST and IGF-I in the present study. The *in vitro* pST experiment in the present study confirmed the results of the previous *in vitro* study, i.e. pST reduces UCP2 mRNA abundance. The present study also indicates that IGF-I may function as an intermediary to produce the *in vivo* UCP2 response. While pST *in vitro* reduced UCP2 mRNA abundance in MSQ and OSQ tissue slices (as in the previous *in vitro* study), IGF-I increased UCP2 mRNA abundance in adipose tissue slices *in vitro*; in agreement with the *in vivo* observations in adipose tissue. We cannot exclude the possibility of cross-reactivity of supplemental IGF-I with the insulin receptor as contributing to this response, although no study has previously demonstrated insulin regulation of adipose UCP2 mRNA abundance. However, the large increase in serum IGF-I with pST treatment as well as the high correlations between serum IGF-I and the mRNA abundance of UCP2 across all of the adipose tissue depots suggests GH induces IGF-I as a mediator of UCP2 expression in adipose tissue *in vivo*.

Presently there are no other studies which have examined a potential role for IGF-I in the regulation of UCP2 expression for any tissue, although Teruel et al. [39] have previously reported that IGF-I can induce the expression of UCP1 in brown adipose tissue *in vitro*. Further research will be necessary to determine whether the reversal in UCP response to 1000 ng pST/mL versus 100 ng/mL may actually reflect paracrine production of IGF-I *in vitro* by the adipose tissue slices which then feeds back on the tissue to up regulate UCP mRNA abundance. Somatotropin has been demonstrated to induce IGF-I expression by porcine adipose cultures [40].

Despite the changes in serum T_3 with pST administration, a general role for T_3 in the regulation of adipose UCP2 could not be identified in the present study, unlike for other species [41]. In support of this observation, *in vitro* dose response experiments could not detect an effect of 0.1–10.0 nM T_3 on UCP2 or UCP3 mRNA abundance in adipose tissue explants (data not presented), in agreement with previous observations from porcine adipose tissue explants [13]. Leaf fat, an internal location of adipose tissue deposition, was the only adipose tissue examined to correlate to UCP2 mRNA abundance with serum T_3. In addition, it was the only adipose tissue examined in which UCP2 mRNA abundance correlated with leptin or NEFA. This exemplifies the variation in regulation of gene expression among the sites of adipose tissue deposition previously reported for swine [42].

There have been no studies that have examined the role of somatotropin in regulation of hepatic uncoupling proteins in any species. The present data would indicate pST, and/or the changes in the concentrations of insulin, IGF-I and NEFA that pST treatment produces, have a role in UCP2 regulation. For example, pST treatment increased serum NEFA in the present study, presumably leading to an increased flux of fatty acids through the liver, as somatotropin has previously been shown to increase hepatic fatty acid uptake in rats [43]. An increase in fatty acid uptake and metabolism has been

shown to stimulate PPARα expression [44]. Thus, an increase in hepatic PPARα mRNA abundance in the present study may contribute to the up regulation of UCP2 expression, as the gene encoding UCP2 contains PPAR response elements in the promoter region [19]. The parallel increase in ACOX mRNA abundance in the liver reflects the regulatory role that PPARα has for this enzyme, since the ACOX gene also contains a PPAR response element [45]. The ACOX enzyme has an essential role in the peroxisomal oxidation of fatty acids in response to metabolic shifts [46], while UCP2 has been suggested to function in the metabolism of peroxides [47].

The present study did not detect an effect of pST on LM UCP2 mRNA abundance, in agreement with Hioki et al. [18] who reported no change in skeletal muscle UCP2 mRNA abundance in lean mice injected with somatotropin. However there may be a muscle specific response as Pedersen et al. [16] reported that UCP2 mRNA abundance was slightly depressed in the vastus lateralis by somatotropin treatment of somatotropin deficient humans. Further research with muscles of varying fiber types may be needed to determine whether pST can alter UCP2 mRNA abundance in skeletal muscle.

However, UCP3 in skeletal muscle was affected by pST treatment. This elevation in UCP3 was highly correlated with serum IGF-I and T_3. Mostyn et al. [48] reported that serum IGF-I correlated with skeletal muscle UCP3 mRNA abundance in neonatal swine. Acute T_3 treatment has been shown to produce an increase in UCP3 mRNA abundance in selected muscles in neonatal pigs [11] and in rats [49]. The potential chronic elevation in T_3 produced by pST treatment has been previously reported [35,36] and confirmed in this study. Thus, the increases in IGF-I and T_3 with pST treatment observed in the present study may contribute to the increase in LM UCP3 mRNA abundance.

Serum NEFA and triglycerides were also correlated with UCP3 mRNA abundance in LM. Unsaturated fatty acids have been shown to induce UCP3 in C_2C_{12} myotubes [50] and in human skeletal muscle [20]. Fasting produces an elevation in serum NEFA and has been associated with an increase in UCP3 mRNA abundance in skeletal muscle of swine [12]. Sbraccia et al. [20] demonstrated that fatty acid induction of UCP3 was through binding to PPARγ, while Muoio et al. [21] reported that the effect occurs through binding to PPARδ. The present study demonstrated that both PPARα and PPARγ mRNA abundance are depressed in skeletal muscle by pST treatment of swine while PPARδ was elevated in association with the increase in serum NEFA and muscle UCP3. Thus, the present data suggest that the mechanism of growth hormone action on fatty acid induction of skeletal muscle UCP3 is through PPARδ. However, we cannot exclude a specific role for either PPARγ1 or PPARγ2 as we measured the combination.

Relating the changes in the mRNA abundance for UCP2 and UCP3 in swine is difficult as no antibodies exist for quantitative measurement of UCP protein levels. No UCP3 antibody has been demonstrated to be effective for swine UCP3 [51] and the only effective UCP2 antibody [51] has with further propagation been characterized as not cross-reacting well with the pig [48]. Therefore, the relationship between changes in UCP mRNA abundance with pST treatment and potential changes in UCP protein concentration cannot be ascertained. Previous studies have demonstrated that UCP2 and UCP3 mRNA and protein levels do not necessarily correlate [51,52]. Therefore, caution must be used in interpreting the relationship between changes in porcine UCP mRNA abundance and UCP activity.

Irrespective of the relationship between mRNA and protein levels, this study demonstrates that pST has selective *in vivo* effects on the UCP2 and UCP3 mRNA abundance in porcine tissues. A variety of endocrine and metabolic adaptations to pST treatment may contribute to these changes in gene expression which are tissue specific. If these changes in UCP mRNA are related to changes in the activity of the uncoupling proteins, the question remains as to what the implications are.

Identification of the functions of the uncoupling proteins has been an area of great interest in the past several years [6,53,54]. Various studies have proposed that UCP2 functions in reducing reactive oxygen species that are generated by mitochondria with metabolic activity [55,56], although this has recently been challenged [57]. Further research has also identified a role for UCP2 in reducing the consequent lipoperoxidation within mitochondria [47]. In speculation, the UCP2 response by liver and adipose tissues in the present study may suggest a potential role for porcine UCP2 in reducing the concentration of somatotropin-induced reactive oxygen species, as suggested by the reduced levels of various enzymes which catalyze reactive oxygen species with GH treatment of rat hepatocytes [15].

Meanwhile, UCP3 has also been associated with reducing reactive oxygen species in skeletal muscle [58] but has also been associated with the intracellular transport of fatty acid anions from the mitochondrial matrix to the cytosol [59]. The flux of fatty acids induces an increase in PPARδ with subsequent induction of UCP3 [60,61], as supported by the present study. Thus UCP3 may form a component of a fatty acid–fatty acyl CoA cycle across the mitochondrial membrane, providing

mitochondrial CoASH for fatty acid oxidation reactions [62,63]. The increase in UCP3 mRNA abundance may reflect an increase in muscle fatty acid metabolism, with the partitioning of consequent energy toward protein synthesis with pST treatment.

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